Endonuclease G is an apoptotic DNase when released from mitochondria

Lily Y. Li, Xu Luo & Xiaodong Wang

Howard Hughes Medical Institute & Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, USA

Nucleosomal fragmentation of DNA is a hallmark of apoptosis (programmed cell death)[1], and results from the activation of nucleases in cells undergoing apoptosis. One such nuclease, DNA fragmentation factor (DFF, a caspase-activated deoxyribonuclease (CAD) and its inhibitor (ICAD)), is capable of inducing DNA fragmentation and chromatin condensation after cleavage by caspase-3 (refs 2–4). However, although transgenic mice lacking DFF45 or its caspase cleavage site have significantly reduced DNA fragmentation[5,6], these mice still show residual DNA fragmentation and are phenotypically normal[5–7]. Here we report the identification and characterization of another nuclease that is specifically activated by apoptotic stimuli and is able to induce nucleosomal fragmentation of DNA in fibroblast cells from embryonic mice lacking DFF. This nuclease is endonuclease G (endoG), a mitochondrion-specific nuclease that translocates to the nucleus during apoptosis. Once released from mitochondria, endoG cleaves chromatin DNA into nucleosomal fragments independently of caspases. Therefore, endoG represents a caspase-independent apoptotic pathway initiated from the mitochondria.

After the activation of cell-surface death receptors such as Fas/CD95, caspase-8 cleaves Bid, thus freeing the truncated Bid (tBid) to translocate to the mitochondria and release apoptogenic factors such as cytochrome *c*, which in turn triggers the activation of caspase-9 (refs 8–10). This reaction has been reconstituted *in vitro* with purified mitochondria, recombinant Bid and recombinant caspase-8. Using this system, we looked for additional apoptogenic factors, particularly DNase activities released from the mitochondria, by incubating the supernatant of Bid plus caspase-8 treated mitochondria with isolated nuclei from normal HeLa cells. As shown in Fig. 1, the nuclei incubated with the supernatant from untreated mitochondria (lane 1), or with the supernatant from mitochondria treated with either full-length Bid or caspase-8 alone (lanes 2 and 3) showed no detectable DNA fragmentation. In contrast, the supernatants from mitochondria incubated with both caspase-8 and Bid induced DNA fragmentation in the co-incubated nuclei (lane 4). The pre-incubation of a caspase inhibitor (z-Vad-fmk) with caspase-8 abolished the activity of the DNase released from the mitochondria (lane 5), indicating that the generation of tBid is necessary for the release of this activity. However, when we pre-incubated caspase-8 and Bid to generate tBid first, and then incubated the mixture with z-Vad-fmk before adding the mitochondria, the DNA-fragmentation activity was still present in the treated mitochondrial supernatant (lane 6). These results indicate that the release of this DNase from mitochondria is dependent on the generation of tBid, but that the activity of the DNase is independent of caspase once it is released from mitochondria.

Using DNA fragmentation in the co-incubated nuclei as an assay, we fractionated the supernatant of mouse liver mitochondria treated with recombinant Bid and caspase-8, and purified the DNase released from mitochondria with a six-step protocol (see Methods). The result of the last step of purification, a Superdex 200 gel-filtration chromatography column, is shown in Fig. 2. The DNA-fragmentation activity was observed in column fractions 11–13 (upper panel). The same fractions were also subjected to 15% SDS–polyacrylamide gel electrophoresis (PAGE) and the proteins were visualized by silver staining. A protein band from the silver-stained gel with a relative molecular mass of 30,000 (M_r 30K) correlated with the DNA-fragmentation activity (lower panel).

To obtain the sequence identity of this putative DNase, the protein was excised from the gel and subjected to tryptic digestion followed by mass-spectral analysis. The mass fingerprinting of the digested peptides matched that of a known mouse mitochondrial nuclease, endoG (see Supplementary Information). EndoG is encoded by a nuclear gene and translated in the cytosol with the amino-terminal 48 amino acids being the mitochondrial targeting

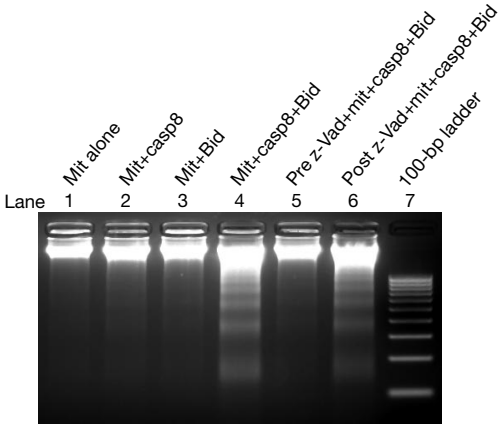

Figure 1 Identification of the mitochondrial nuclease. Isolated mice liver mitochondria (mit) were incubated with buffer alone (lane 1), with purified recombinant Bid or caspase-8 (casp8) separately (lanes 2, 3), or with both recombinant Bid and caspase-8 (lane 4). Reactions were prepared by either pre-incubating 20 μM z-Vad-fmk (z-Vad) with caspase-8 for 15 min at 30 °C before adding Bid and purified mitochondria (lane 5), or pre-incubating with caspase-8 and Bid for 15 min at 30 °C then with 20 μM z-Vad-fmk for 15 min at 30 °C before adding purified mitochondria (lane 6). The reactions were then incubated and centrifuged (see Methods) and the supernatants were collected. Aliquots of 38 μl of the supernatant fluid were incubated with 17 μl of buffer D (buffer A, 10 mM $MgCl_2$ and 100 μM $CaCl_2$) and 5 μl of purified nuclei for 2 h at 37 °C and purified (see Methods). Samples of the resuspended DNA were electrophoresed on a 1.2% agarose gel in 0.5 × TBE (45 mM Tris-HCl at pH 8.0, 45 mM boric acid, 1 mM EDTA) after RNAse treatment.

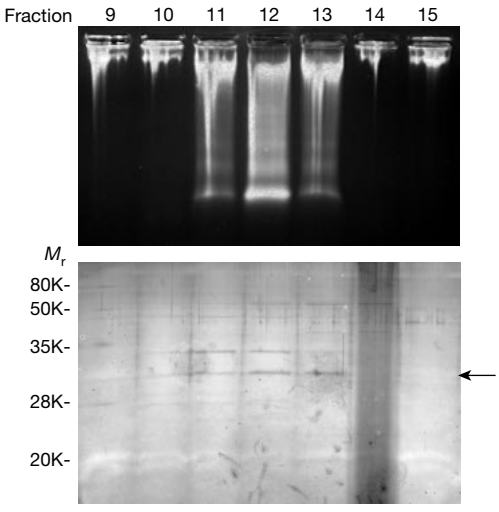

Figure 2 Purification of the mitochondrial nuclease (arrowed). Aliquots from the last purification step, the Superdex 200 column, were assayed for DNA fragmentation activity by incubation with isolated nuclei (upper panel; see Methods). The same samples were also subjected to 15% SDS–PAGE and silver stained (lower panel).

sequence, which is subsequently removed when the protein is imported into mitochondria[11,12].

To confirm that the DNase released from mitochondria by tBid is endoG, we generated a polyclonal antibody against the bacterially expressed endoG (amino acids 127–286) and performed western blotting and immunodepletion experiments with this antibody (Fig. 3a). This antibody specifically recognizes the precursor and the mature forms of both recombinant human endoG generated with a baculovirus expression system and murine endoG in mouse liver mitochondria (middle panel, lanes 1 and 2, respectively). The mature recombinant human endoG is slightly larger than mouse endoG possibly owing to the different processing in insect cells, the presence of a six-histidine tag, and additional amino acids. The pre-immune serum from the same animal failed to recognize these two bands (Fig. 3a, left panel). To further prove the specificity of the antibody, we showed that the addition of recombinant endoG during western blot can block the immunoreactivity toward these two bands (right panel). The depletion of endoG from the tBid-treated mitochondrial supernatant with the anti-endoG antibody eliminated the DNA fragmentation (Fig. 3b, upper panel, lane 6) as well as the plasmid-cleaving DNase activity (lower panel, lane 6), indicating that endoG is the only DNase present in the tBid-treated mitochondrial supernatant. In contrast, the same depletion experiment performed with the pre-immune serum did not deplete the DNase activity (lane 5).

We next tested whether endoG alone is sufficient to cleave nuclear DNA into a discrete ladder using the purified recombinant endoG. As shown in Fig. 3c, increasing amounts of purified recombinant endoG induced increasing levels of DNA fragmentation in the co-incubated nuclei and cleaved plasmid DNA into smaller fragments. These results indicate that endoG is sufficient to generate DNA fragmentation.

We also compared the time course of release for both endoG and cytochrome *c* induced by purified tBid using a mitochondrial matrix protein, heat shock protein 70 (mtHsp70), as a control. As shown in Fig. 4, in the absence of tBid, neither cytochrome *c* nor endoG was released from mitochondria even after incubation for 90 min (lane 9). In the tBid-treated mitochondria, both endoG and cytochrome *c* were observed in the mitochondrial supernatants after 15 min of incubation (lane 4). By 90 min, most of the cytochrome *c* and endoG were released from mitochondria (lane 10). In contrast, the mtHsp70 was not released from mitochondria, indicating the inner membrane of mitochondria remained intact.

To test whether endoG is released in apoptotic cells, we performed immunofluorescent staining of both normal and apoptotic cells (Fig. 5). We chose mouse embryonic fibroblast (MEF) cells from the DFF45/ICAD-knockout mice for this study because these cells have lost the activities of caspase-3-dependent DNA fragmentation and chromatin condensation[5]. Immunostaining of untreated DFF45/ICAD-knockout MEF cells with the anti-endoG antibody revealed a

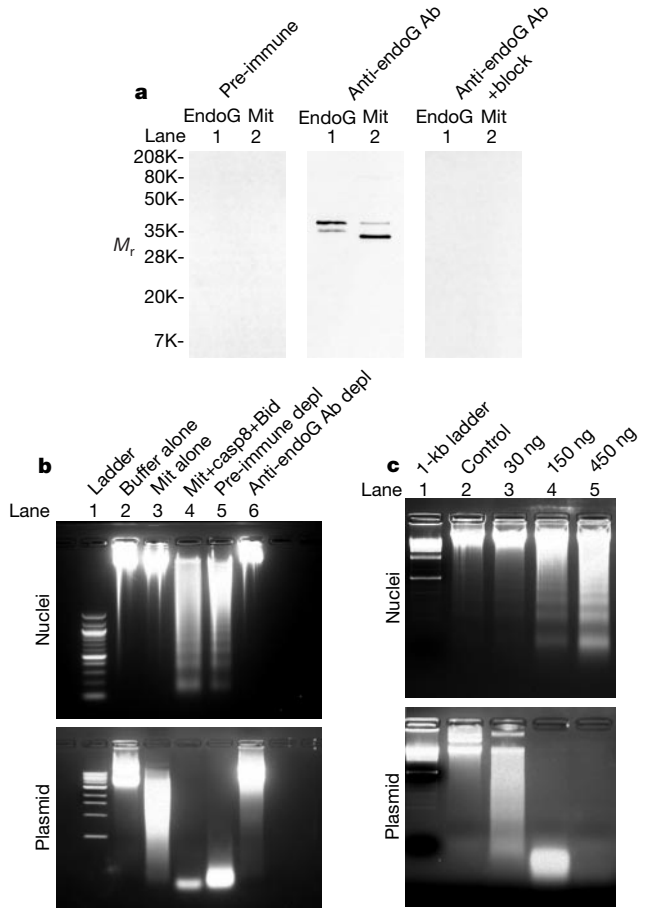

Figure 3 EndoG is both necessary and sufficient to cause DNA fragmentation. **a**, Recombinant human endoG and mouse mitochondrial pellet (Mit) were resuspended in 1× SDS loading buffer and subjected to 15% SDS–PAGE. The samples were transferred to a nitrocellulose membrane and western blots were performed using either pre-immune samples, anti-endoG antibodies (Ab) or endoG antibodies in the presence of recombinant endoG, as indicated. **b**, Isolated mice liver mitochondria were incubated with buffer alone or with recombinant Bid and caspase-8 (casp8). After centrifugation, aliquots of the supernatant from the treated mitochondria were immunodepleted (depl) with protein A-agarose beads coated either with control pre-immune antibodies or anti-endoG antibodies, and the DNA-fragmentation assay with isolated nuclei was performed. Resuspended DNA from the assays was visualized after electrophoresis on a 1.2% agarose gel in 0.5 × TBE gel. **c**, Recombinant human endoG was prepared (see Methods) and the protein concentrations were determined with the Bradford assay. Titration of the purified recombinant protein with both nuclear fragmentation and plasmid cleavage assays was performed to test the nuclease activity of the recombinant protein. The resulting samples were loaded onto a 1.2% agarose gel in 0.5 × TBE gel.

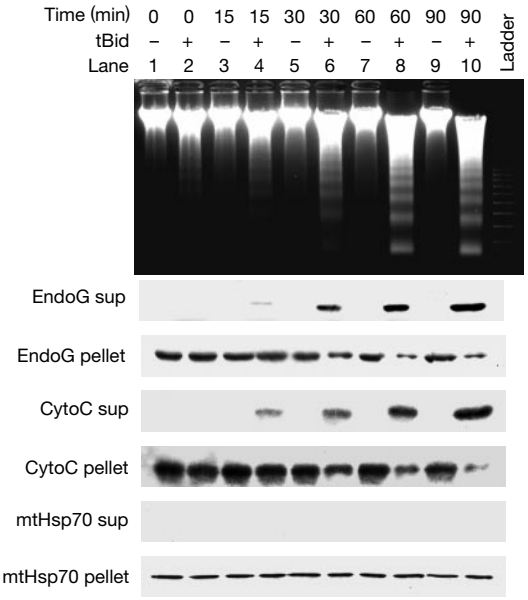

Figure 4 Characterization of the release process of endoG. Isolated mitochondria from mouse liver were incubated with either control buffer or purified recombinant tBid as described[16] for up to 90 min at 30 °C. Aliquots were taken at each time point as indicated and subjected to centrifugation at 12,000*g* for 15 min. The resulting supernatants (sup) were used for DNA-fragmentation activity (upper panel) and western blot analysis using antibodies against cytochrome *c* (CytoC), endoG, and mtHsp70. The resulting mitochondrial pellets were subjected to western blot analysis using antibodies against these three proteins.

punctate mitochondrial pattern that co-localizes with cytochrome *c*. The staining of endoG but not cytochrome *c* was blocked by the addition of recombinant endoG. Upon induction of apoptosis by ultraviolet irradiation or treatment in TNF with cyclohexamide (CHX), both endoG and cytochrome *c* were released from the mitochondria to cytosol and nuclei.

The immunostaining data indicate that the DFF-knockout cells might still fragment their DNA, albeit at a reduced level when undergoing apoptosis, possibly owing to the release of endoG from mitochondria. To test this possibility, we analysed DNA fragmentation in the wild-type and DFF45-knockout MEF cells undergoing apoptosis induced by ultraviolet irradiation and TNF with CHX. As shown in Fig. 6a, both ultraviolet irradiation and TNF/CHX treatment induced DNA fragmentation in wild-type and DFF-knockout MEF cells, although the wild-type cells showed more DNA fragmentation (lanes 5, 6, 9 and 10). When we pre-incubated cells with media containing z-Vad-fmk before ultraviolet treatment, the level of DNA fragmentation decreased in the wild-type cells but was largely unaffected in the DFF-knockout cells (lanes 7 and 8). In contrast, z-Vad-fmk essentially eliminated the ability of TNF/CHX to induce DNA fragmentation in both the wild-type and the DFF-knockout MEF cells (lanes 11 and 12). This finding again confirmed that caspase-8 activation in TNF-induced apoptosis is upstream of the mitochondrial damage and the release of apoptogenic factors[13]. In contrast, caspase activation occurs after mitochondrial damage and cytochrome *c* release following ultraviolet irradiation[14]. Therefore the caspase inhibitor can inhibit DNA fragmentation caused by DFF, which has an absolute dependence on activated caspase for its activity, but can not inhibit the release of endoG from mitochondria following ultraviolet irradiation.

To confirm that the DNA-fragmentation activity observed in the DFF45-knockout MEF cells is caused by endoG, we prepared extracts containing both nuclei and mitochondria from the DFF45-knockout MEF cells and induced the fragmentation of chromatin in the nuclei with tBid. As shown in Fig. 6b, addition of tBid induced nucleosomal fragmentation in the nuclei prepared from DFF45-knockout cells (lane 2). Pre-incubation of tBid with about twofold more of the death-preventing protein Bcl-x_L completely blocked the DNA fragmentation, indicating that the DNA fragmentation was caused by factors released from the co-incubated mitochondria and that the release process was regulated by Bcl-x_L (lane 3). Addition of the polyclonal antibody against endoG in the reaction also completely blocked DNA fragmentation (lane 5) while the pre-immune serum showed no effect (lane 4).

The persistence of DNA fragmentation induced by ultraviolet irradiation in the presence of caspase inhibitor indicated that DNA fragmentation could be induced by endoG independently of caspase. To further demonstrate this point, we used another pro-apoptotic protein, Bim, to repeat the experiment shown in Fig. 6b. Like Bid, Bim is a member of the 'BH3-only' pro-death family of proteins and can release cytochrome *c in vitro* as efficiently as tBid (data not shown). Unlike Bid, the apoptotic activity of Bim is regulated through its dissociation from microtubules during apoptosis in a caspase-independent fashion[15]. As shown in Fig. 6c, purified recombinant Bim fused to glutathione *S*-transferase (GST) induced DNA fragmentation in extracts prepared from DFF-knockout cells (lane 2) whereas GST protein had no effect (lane 6). As with tBid, both Bcl-x_L and the antibody against endoG

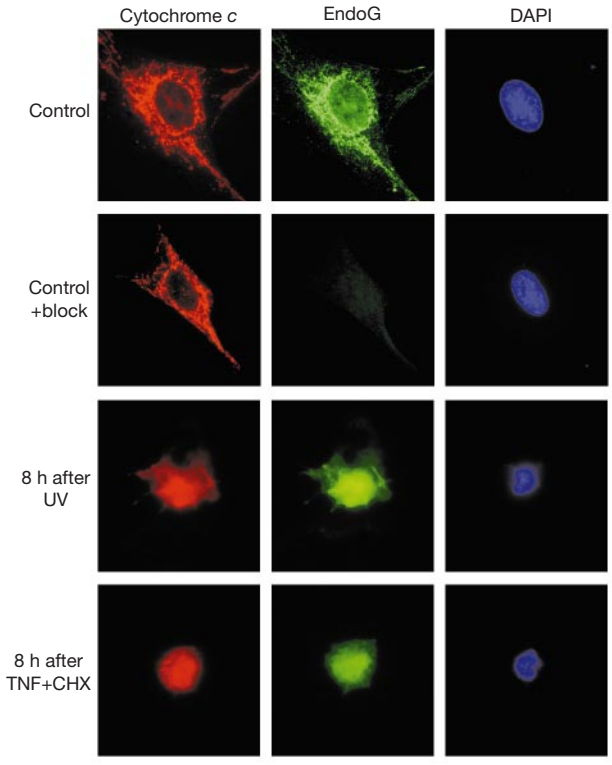

Figure 5 EndoG is released from mitochondria of apoptotic cells during apoptosis. Immunostaining of both endoG and cytochrome *c* (see Methods) was performed on untreated control cells and cells treated with ultraviolet (UV) radiation or TNF/CHX. Control cells were also stained with cytochrome *c* and anti-endoG antibody in the presence of recombinant endoG (block). Cell nuclei were stained with DAPI.

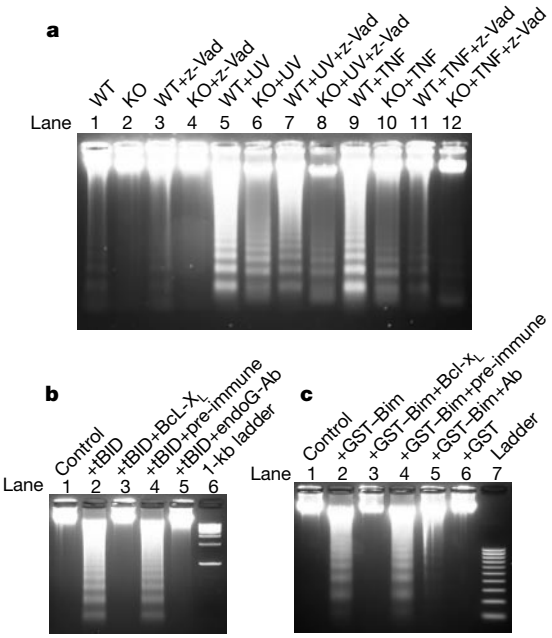

Figure 6 DFF-knockout cells can undergo caspase-independent DNA fragmentation. **a**, Wild-type (WT) and DFF-knockout (KO) MEF cells were treated with ultraviolet radiation or TNF/CHX, respectively (see Methods). z-Vad-fmk (z-Vad) was added to the medium to 50 μM 4 h before treatment. Cells were collected 24 h after the treatment and processed (see Methods). Aliquots of 20 μg of the extracted genomic DNA were subjected to 1.2% TBE agarose gel electrophoresis and visualized under ultraviolet light. **b**, **c**, DFF-knockout cells (2×10^6) were collected by trypsinization and washed three times with cold PBS. The pellet was resuspended in 1 ml of buffer L (20 mM HEPES at pH 7.5, 10 mM KCl, 4 mM $MgCl_2$, 0.1 mM PMSF and 250 mM sucrose), processed 10 times with a teflon Dounce homogenizer and centrifuged at 12,000*g* for 15 min at 4 °C, The pellet, containing both nuclei and mitochondria, was resuspended in 400 μl of buffer L. Aliquots of 53 μl were then added to the following: buffer L alone (control, **b** and **c**), 400 ng of tBid (**b**) or 600 ng of GST–Bim (**c**); lane 3 was pre-incubated with 800 ng of Bcl-x_L for 15 min at 37 °C, lane 4 included 2 μl of pre-immune serum, and lane 5 included 2 μl of anti-endoG antibody (Ab); lane 6 was 600 ng of GST alone. The reactions were incubated at 37 °C for 3 h and the DNA samples were isolated and analysed (see Methods).

efficiently blocked Bim-induced DNA fragmentation while pre-immune serum did not (lanes 3–5).

These experiments revealed endoG as another apoptogenic protein from mitochondria that directly leads to nucleosomal DNA fragmentation. EndoG may serve as an alternative pathway to DFF/CAD to cause genomic DNA fragmentation in a caspase-independent manner.

EndoG has been proposed to participate in mitochondrial replication through the generation of RNA primers required for the initiation of mitochondrial DNA synthesis, an event that takes place in the matrix of the mitochondria[12]. However, because tBid induces release of cytochrome *c* from mitochondria without affecting the mitochondrial membrane potential, and because a mitochondrial matrix protein, mtHsp70, was not released by tBid even when most of the cytochrome *c* and endoG had been released, it is likely that most of the endoG co-localizes with cytochrome *c* in the intermembrane space (ref. 16 and Fig. 4). Also consistent with its proposed function in apoptosis, the treatment of DNA with damaging agents such as L-ascorbic acid, peplomycin and cisplatin could enhance the susceptibility of DNA to the nuclease activity of endoG[17]. Such a property may be important for endoG to process damaged DNA that may be more resistant to other DNases.

Support has been increasing for the idea that, even though caspases are important in many aspects of programmed cell death, cells can still undergo programmed cell death without caspase activation. The broad-spectrum caspase inhibitor z-Vad-fmk could not prevent cell death induced by the overexpression of Bax to mitochondria even though most of the caspase-mediated apoptotic markers were eliminated[18]. The importance of mitochondria was further demonstrated by the identification of several apoptogenic factors. In addition to cytochrome *c*, which activates caspases through its cofactor Apaf-1, mitochondria in cells undergoing apoptosis also release Smac (also known as DIABLO) to counteract the caspase-inhibitory activities of IAPs (inhibitor-of-apoptosis proteins) and AIF (apoptosis-inducing factor) to cause fragmentation of DNA into large sections (M_r ~50K) and condensation of peripheral nuclear chromatin[19–21]. Moreover, mice lacking Bak and Bax displayed striking phenotypes, such as the retention of interdigital web in adult animals and accumulation of excess cells in the haematopoietic system, which were not observed in animals with Apaf-1 or caspase-9 knocked out[23–29]. Examination of the Apaf-1-knockout mice showed necrotic cell death during interdigital web recession without caspase activation[30]. These results indicate that the mitochondrial damage leading to the release of proteins of the intermembrane space might lead to both caspase-dependent and -independent cell death. EndoG represents such a caspase-independent pathway of cell death initiated from mitochondria. □

Methods

In vitro assay for DNase activity

Mouse liver mitochondria, the full-length recombinant caspase-8 and Bid were prepared as described[9]. A 6-μl aliquot of purified mouse liver mitochondria was added to 4 μl of caspase-8 (0.2 mg ml^{-1}) and 1 μl Bid (1 mg ml^{-1}), or 1 μl tBid (0.1 mg ml^{-1}) in a final volume of 50 μl buffer R (20 mM HEPES–KOH at pH 7.5, 220 mM mannitol, 68 mM sucrose, 10 mM KCL, 1.5 mM MgCl2, 1 mM NaEDTA, 1 mM NaEGTA, 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethyl sulphonyl fluoride (PMSF)). After incubation at 30 °C for 45 min and centrifugation at 12,000*g* for 15 min, the supernatants from the reactions were used in either the nuclei or plasmid assay. For the nuclear assay, an aliquot of 38 μl of the supernatant was added to 5 μl of purified nuclei made as described[2].

Purification of the supernatant DNase

Liver mitochondria from 30 Swiss Webster mice were prepared and treated with tBid as described above; the treated-mitochondria supernatant (TMS) was used as the starting material for the following purification procedures, with ammonium sulphate precipitation as the first step. Supernatant from 20% precipitation was further precipitated at a final concentration of 60% ammonium sulphate. The pellet from the precipitation was resuspended in 6 ml of buffer A (20 mM HEPES–KOH at pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.1 mM PMSF), loaded onto a Superdex 200 gel-filtration 10/30 column, equilibrated, and eluted with buffer A. Active DNase fractions were pooled and loaded onto a Mono Q 5/5 column equilibrated with buffer A. The flow-through from the column was collected and loaded onto a Mono S 5/5 column and eluted with a linear gradient of 15 ml from buffer A to buffer A containing 250 mM NaCl. An aliquot of 0.5 ml of the active peak fraction was dialysed against buffer A for 2 h and loaded onto the Superdex 200 column equilibrated and eluted with 60 mM NaCl in buffer A. Fractions of 1 ml were collected and assayed for DNase activity.

Protein identification

A 50-μl aliquot of the active peak fraction from the last purification step was subjected to 15% SDS–PAGE. After staining with silver (Silver-Staining Plus, Bio-Rad), the 30K band that corresponded with the activity was excised from the gel and digested with trypsin. A 1-μl aliquot from the digest was used for peptide mass fingerprinting as described[9]. Mass values were searched against the NCBI database with the program MS-Fit. The mass values of five peptides (a total of 54 amino acids) matched that of mouse endoG with a standard deviation of <0.34.

Production of recombinant proteins

Full-length human endoG with a six-histidine carboxy-terminal tag was generated as described[19] except that the cell pellet was resuspended and sonicated in buffer A with 0.5% CHAPS. A COOH terminal portion (amino acids 127–286) of mouse endoG with a six-histidine C-terminal tag was cloned into pET-15b and the recombinant protein was purified following the protocol described[2]. Full-length Bcl-x_L complementary DNA was cloned into *Nde*I/*Xho*I sites of the expression vector pET-15b and the recombinant protein was purified as above. Full-length GST-tagged Bim-EL was cloned into *Eco*RI/*Xho*I sites of the expression vector pGEX-VP (Novagen) and the recombinant protein was purified using the standard purification protocol for GST-tagged proteins.

Immunodepletion, blotting and immunostaining

Anti-endoG antibodies were generated by immunizing rabbits with the recombinant protein containing the C-terminal portion of endoG prepared as described above. Western blotting and immunodepletion were performed following the protocol described[2]. For immunostaining, DFF45/ICAD-knockout MEF cells were seeded on chamber slides (Nalge Nunc International) in DMEM medium with 10% (v/v) fetal calf serum, and grown overnight in a 37 °C incubator with 5% CO_2. The cells were either irradiated 3.5 cm from an ultraviolet lamp (5 mW cm^{-2}; Philips, G36T6L) or incubated with 2 ng ml^{-1} of TNF-α (Sigma) plus 1 μg ml^{-1} of CHX (Sigma). After 8 h, the cells were immunostained as described[20].

Received 20 February; accepted 23 May 2001.

1. Wyllie, A. H. Glucocorticoid induced thymocyte apoptosis is associated with endogeneous endonuclease activation. *Nature* **284,** 555–556 (1980).
2. Liu, X. *et al.* DFF, heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89,** 175–184 (1997).
3. Liu, X. *et al.* DFF40 induces DNA fragmentation and chromatin condensation during apoptosis. *Proc. Natl Acad. Sci. USA* **15,** 8461–8466 (1998).
4. Enari, M. *et al.* A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391,** 43–50 (1998).
5. Zhang, H. *et al.* Resistance to DNA fragmentation and chromatin condensation in mice lacking the DNA fragmentation factor 45. *Proc. Natl Acad. Sci. USA* **95,** 12480–12485 (1998).
6. McIlroy, D. *et al.* An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. *Genes Dev.* **14,** 549–558 (2000).
7. Zhang, J. H. & Xu, M. DNA fragmentation in apoptosis. *Cell Res.* **10,** 205–211 (2000).
8. Li, H. *et al.* Cleavage of Bid by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94,** 615–624 (1998).
9. Luo, X. *et al.* Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptor. *Cell* **94,** 481–490 (1998).
10. Gross, A. *et al.* Caspase cleaved BID targets mitochondria and is required for cytochome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.* **274,** 1156–1163 (1999).
11. Tiranti, V. *et al.* Chromosomal localization of mitochondrial transcription factor A (TCF6), single-stranded DNA-binding protein (SSBP), and endonuclease G (ENDOG), three human housekeeping genes involved in mitochondrial biogenesis. *Genomics* **25,** 559–564 (1995).
12. Cote, J. & Ruiz-Carrillo, A. Primers for mitochondrial DNA replication generated by endonuclease G. *Science* **261,** 765–769 (1993).
13. Krammer, P. H. CD95's deadly mission in the immune system. *Nature* **407,** 789–795 (2000).
14. Kluck, R. M. *et al.* The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275,** 1132–1136 (1997).
15. Puthalakath, H. *et al.* The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol. Cell* **3,** 287–296 (1999).
16. Von Ahsen, O. *et al.* Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. *J. Cell Biol.* **150,** 1027–1036 (2000).
17. Ikeda, S. & Ozaki, K. Action of mitochondrial endonuclease G on DNA damaged by L-asorbic acid, peplomycin, and cis-Diamminedichloroplatinum (II). *Biochem. Biophys. Res. Commun.* **235,** 291–294 (1997).
18. Xiang, J., Chao, D. T. & Korsmeyer, S. J. BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. *Proc. Natl Acad. Sci. USA* **93,** 14559–14563 (1996).
19. Zou, H. *et al.* An APAF-1 cytochrome C multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem.* **274,** 11549–11556 (1999).
20. Du, C. *et al.* Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102,** 33–42 (2000).

21. Verhagen, A. *et al.* Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102,** 43–53 (2000).
22. Susin, S. A. *et al.* Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397,** 441–446 (1999).
23. Lindsten, T. *et al.* The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. *Mol. Cell* **6,** 1389–1399 (2000).
24. Kuida, K. *et al.* Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384,** 368–372 (1996).
25. Hakem, R. *et al.* Differential requirement for caspase 9 in apoptotic pathways *in vivo*. *Cell* **94,** 339–352 (1998).
26. Kuida, K. *et al.* Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94,** 325–337 (1998).
27. Cecconi, F. *et al.* Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94,** 727–737 (1998).
28. Yoshida, H. *et al.* Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* **94,** 739–750 (1998).
29. Honarpour, N. *et al.* Adult Apaf-1-deficient mice exhibit male infertility. *Dev. Biol.* **218,** 248–258 (2000).
30. Chautan, M. *et al.* Interdigital cell death can occur through a necrotic and caspase-independent pathway. *Curr. Biol.* **9,** 967–970 (1999).

Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank J. M. Peters, M. Lutter and M. Fang for their assistance in mitochondrion purification and other techniques; Y. Li and R. Harold for technical support; J. Zhang and M. Xu for providing DFF45-knockout MEF cells. We also thank M. Lutter and X. Jiang for recombinant Bcl-x_L. X.L. is supported by the Leukemia Society of America; X.W. is supported by grants from NIH and the Welch Foundation.

Correspondence and requests for materials should be addressed to X.W. (e-mail: xwang@biochem.swmed.edu).

corrections

Timing of the Last Glacial Maximum from observed sea-level minima

Yusuke Yokoyama, Kurt Lambeck, Patrick De Deckker, Paul Johnston & I. Keith Fifield

Nature **406,** 713–716 (2000).

In this Letter, a statement on page 715 that the eustatic sea level $\Delta\zeta_{eus}(t)$ differs from the ice-volume-equivalent sea level $\Delta\zeta_e(t)$ is erroneous. We thank W. R. Peltier for drawing this error in interpretation to our attention. The two definitions were initially introduced to distinguish changes in sea level that were the result of ice mass being added to the oceans from other changes caused by thermal expansion, for example. The erroneous statement was the result of a programming error that was introduced into the program in 1998 when it was modified to deal with shelf ice more accurately and to introduce the time dependence of the coastlines in the evaluation of the equivalent sea-level change through equation (3). This evaluation requires a knowledge of the location of the shoreline at each time step, of the grounding line of the ice and of the part of the shelf ice that floats or grounds as a result of sea level change and shelf-ice thickness change. The error was introduced at the end of each iteration of the sea-level equation, when the value of sea level change was integrated over the ocean and the resulting volume was compared with the change in ice volume for the corresponding interval. The eustatic sea level at this stage was defined as the global average of sea-level change but, because we allow sea level to be non-zero on land so as to be able to compute the tilting of lakes or the changing elevations of tree lines, the averaging should have been restricted to the ocean. Fortunately, the error does not enter into any other part of the model predictions because the estimate of global sea level rise is based on the ice volume change in equation (3) throughout the core program. Thus calculations of the isostatic correction $\Delta\zeta_i(j,t)$ in equation (1) and of the ice-volume-equivalent sea level $\Delta\zeta_e(t)$ are correct, as are the results illustrated in Fig. 2. The matter arises in the two sentences in parentheses of the penultimate paragraph of the main text (page 715), in a discussion of the difference between our Barbados results and those of Peltier. The first sentence in parentheses remains correct, but our attempt to explain it in the second sentence is not. The cause for the disagreement must be sought elsewhere, possibly in the different ice and/or earth models used. □

The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer

Emad M. El-Omar, Mary Carrington, Wong-Ho Chow, Kenneth E. L. McColl, Jay H. Bream, Howard A. Young, Jesus Herrera, Jolanta Lissowska, Chiu-Chin Yuan, Nathaniel Rothman, George Lanyon, Maureen Martin, Joseph F. Fraumeni Jr & Charles S. Rabkin

Nature **404,** 398–402 (2000).

We reported the association of interleukin-1 gene cluster polymorphisms suspected of enhancing production of interleukin-1 β with an increased risk both of hypochlorhydria induced by *Helicobacter pylori* and of gastric cancer. We were subsequently alerted to an error in our genotyping by a report[1] that the *IL-1B*-511T variant is in positive linkage disequilibrium with the *IL-1B*-31C allele, rather than with the *IL-1B*-31T allele as we had stated. We have traced this discrepancy to incorrect labelling of the sequence data from the wild-type and variant controls for the *IL-1B*-31 assays. Consequently, the designations *IL-1B*-31T and *IL-1B*-31C were reversed in the text and Tables 1–3. We have now verified the proper identifications by re-sequencing our controls. In addition, we have confirmed the more common (*IL-1B*-31T/*IL-1B*-511C) and less common (*IL-1B*-31C/*IL-1B*-511T) haplotypes by forward and reverse sequencing five homozygous samples (two wild-type and three variant) for both of these loci. Our data and analysis remain otherwise unchanged. The electrophoretic mobility shift assays were unaffected by this error and hence the greater DNA binding of the *IL-1B*-31T-bearing oligonucleotide represents the wild-type *IL-1B* promoter. □

1. Hamajima, N. *et al.* Interleukin-1 polymorphisms, lifestyle factors, and *Helicobacter pylori* infection. *Jpn J. Cancer Res.* **92,** 383–389 (2001).